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(54) Title: HERPES SIMPLEX VACCINE COMPRISING HSV GLYCOPROTEIN gD AND 3 DEACYLATED MONOPHOSPHORYL LIPID A (57) Abstract Novel herpes simplex (HSV) vaccine formulations are provided. These comprise HSV glycoprotein gD or immunological fragments in conjunction with 3 Deacylated monophosphoryl lipid A.		

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Herpes simplex vaccine comprising HSV glycoprotein GD and
3 Deacylated monophosphorge Lipid A.

The present invention relates to novel vaccine formulations, methods for
preparing them and to their use in therapy. In particular, the present
5 invention relates to novel formulations for treating Herpes Simplex Virus
infections, more particularly Herpes Simplex virus 2 (HSV-2) infections.

HSV-2 is the primary etiological agent of herpes genitalis and together
with HSV-1 (the causative agent of herpes labialis) are characterised by
10 their ability to induce both acute diseases and to establish a latent
infection, primarily in neuronal ganglia cells.

Genital herpes is estimated to occur in about 5 million people in the
U.S.A. alone with 500,000 clinical cases recorded every year (primary and
15 recurrent infection),. Primary infection typically occurs after puberty and
is characterised by the localised appearance of painful skin lesions, which
persist for a period of between 2 to 3 weeks. Within the following six
months after primary infection 50% of patients will experience a
recurrence of the disease. About 25% of patients may experience between
20 10-15 recurrent episodes of the disease each year. In
immunocompromised patients the incidence of high frequency recurrence
is statistically higher than in the normal patient population.

Both HSV-1 and HSV-2 virus have a number of glycoprotein components
25 located on the surface of the virus. These are known as gA, gB, gC, gD
and gE etc.

Glycoprotein D is located on the viral membrane, and is also found in the
cytoplasm of infected cells (Eisenberg R.J. *et al*; J of Virol 1980 35 428-
30 435). It comprises 393 amino acids including a signal peptide and has a
molecular weight of approximately 60 kD. Of all the HSV envelope
glycoproteins this is probably the best characterised (Cohen *et al* J.
Virology 60 157-166). *In vivo* it is known to play a central role in viral
attachment to cell membranes. Moreover, glycoprotein D has been shown
35 to be able to elicit neutralising antibodies *in vivo* (Eing *et al* J. Med.
Virology 127: 59-65). However, latent HSV-2 virus can still be reactivated
and induce recurrence of the disease despite the presence of high
neutralising antibodies titre in the patients sera.

The ability to induce neutralising antibody alone is insufficient to adequately control the disease. In order to prevent recurrence of the disease, any vaccine will need to stimulate not only neutralising antibody,
5 but also cellular immunity mediated through T-cells. The present invention achieves these aims.

The present invention provides a vaccine comprising HSV glycoprotein D or an immunological fragment thereof in conjunction with 3-o-deacylated
10 monophosphoryl lipid A (3D-MPL) a deacylated derivative of monophosphoryl lipid A, and a suitable carrier. Typically the glycoprotein D will be from HSV-2. The carrier may be an oil in water emulsion, or alum, 3D-MPL will be present in the range of 10µg - 100µg preferably 25-
15 50µg per dose wherein the antigen will typically be present in a range 2-50µg per dose.

3D-MPL may be obtained according to the methods described in British patent No. 2220211 (RIBI).

20 An embodiment of the invention is a truncated HSV-2 glycoprotein D of 308 amino acids which comprises amino acids 1 through 306 naturally occurring glycoprotein with the addition Asparagine and Glutamine at the C terminal end of the truncated protein devoid of its membrane anchor region. This form of the protein includes the signal peptide which is
25 cleaved to yield a mature 283 amino acid protein. The production of such a protein in Chinese Hamster ovary cells has been described in Genentech's European patent EP-B-139 417.

30 The mature truncate preferably is used in the vaccine formulations of the present invention as is designated rgD_{2t}.

The HSV antigen may be chemically or otherwise conjugated to a particulate carrier. A particularly preferred approach is to chemically conjugate to particulate Hepatitis B surface antigen through free
35 sulphhydryl groups located on the surface of the Hepatitis B surface antigen. See copending U.K. Patent application No. 9027623.9.

The formulations of the present invention are very effective in inducing

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protective immunity, even with very low doses of antigen (e.g. as low as 5 μg rgD₂t).

5 They provide excellent protection against primary infection and stimulate, advantageously both specific humoral (neutralising antibodies) and also effector cell mediated (DTH) immune responses.

Non-toxic oil in water emulsions preferably contain a non-toxic oil, e.g. squalane or squalene, a emulsifier, e.g. Tween 80, in an aqueous carrier.
10 The aqueous carrier may be for example, phosphate buffered saline.

The present invention in a further aspect provides a vaccine formulation as herein described for use in medical therapy, particularly for use in the treatment or prophylaxis of Herpes Simplex viral infections.
15

The vaccine of the present invention will contain an immunoprotective quantity of HSV gD or immunological fragment thereof and this maybe prepared by conventional techniques.

20 Vaccine preparation is generally described in New Trends and Developments in Vaccines, edited by Voller et al., University Park Press, Baltimore, Maryland, U.S.A. 1978. Encapsulation within liposomes is described, for example, by Fullerton, U.S. Patent 4,235,877. Conjugation of proteins to macromolecules is disclosed, for example, by Likhite, U.S.
25 Patent 4,372,945 and by Armor et al., U.S. Patent 4,474,757.

The amount of protein in each vaccine dose is selected as an amount which induces an immunoprotective response without significant, adverse side effects in typical vaccinees. Such amount will vary depending upon
30 which specific immunogen is employed. Generally, it is expected that each dose will comprise 1-1000 μg of protein, preferably 2-100 μg , most preferably 4-40 μg . An optimal amount for a particular vaccine can be ascertained by standard studies involving observation of antibody titres and other responses in subjects. Following an initial vaccination, subjects
35 may receive a boost in about 4 weeks.

In addition to vaccination of persons susceptible to HSV infections, the pharmaceutical compositions of the present invention may be used to

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treat, immunotherapeutically, patients suffering from HSV infections.

In a further aspect of the present invention there is provided a method of manufacture as herein described, wherein the method comprises mixing
5 HSV-2 glycoprotein D or an immunological fragment with a carrier, e.g. an oil in water emulsion or alum, and 3D-MPL.

Comparison of adjuvant efficacy of a recombinant Herpes Simplex Virus Glycoprotein D Subunit Vaccine

10

In this study, the ability of several adjuvants to improve the protective immunity of a recombinant glycoprotein D from Herpes Simplex Virus (HSV) type 2 (rgD_{2t}) was evaluated in a guinea pig model. Adjuvants tested were aluminium hydroxide, aluminium hydroxide in combination
15 with 3 Deacyl-Monophosphoryl Lipid A, and 3 Deacyl-Monophosphoryl Lipid A delivered in an oil in water emulsion.

1. Description of the antigen

20 HSV rgD_{2t} is a genetically engineered recombinant truncated glycoprotein produced in transfected Chinese hamster ovary (CHO) cells (European Patent No. 0 139 417).

2. Antigen-Adjuvant preparations and immunization schedules

25

Two separate experiments were performed to evaluate the protective immunity of several rgD_{2t} formulations in the guinea pig model. In the first experiment, groups of guinea pigs were immunized three times with a low antigen dose (5 µg of rgD_{2t}) in 4 adjuvant formulations prepared as
30 described below. Two weeks after the last immunization, they were challenged intravaginally with HSV type 2 and were monitored daily for the development of primary and recurrent HSV2 disease. In the second experiment, these formulations were further evaluated on larger animal groups. Factors influencing efficacy of these formulations were also tested
35 such as antigen dose and adjuvant composition.

2.1. Antigen-Adjuvant preparations

5 In the first experiment, guinea pigs were immunized with the following adjuvant preparations. Each dose (5 µg) was administered in a 0.25 ml volume.

2.1.1. rgD2t/Alum (Aluminium Hydroxide)

10 Alum was obtained from Superfos (Alhydrogel, (Boehimte) Superfos, Denmark). Five µg of purified rgD2t was adsorbed overnight at 4°C on aluminium hydroxide (alum) corresponding to 0.25 mg equivalents Al^{3+} in 0.25 ml of 150 mM NaCl 10 mM phosphate buffer pH 6.8.

2.1.2. rgD2t / Aluminium Hydroxide plus 3D-MPL

15 3 D-MPL was obtained from Ribi Immunochem Research, Inc. After an overnight adsorption of 5 µg gD2t on alum as described in 2.1.1., the adjuvant preparation was centrifuged and its supernatant removed. An equal volume of adsorption buffer containing 100 µg 3D-MPL was then
20 added to the alum-bound rgD2t.

For both rgD2t/Alum preparations, more than 98% of the rgD2t was found to be incorporated in aluminium hydroxide adjuvant.

2.1.3. rgD2t/3D-MPL in an oil in water emulsion (R)

25 The oil in water emulsion was prepared using 12% w/v lecithin added to Squalene oil and 0.08% Tween 80. 3D-MPL was added at a concentration 100 fold higher than the final desired concentration. 1% of this preparation was then mixed in a 0.25 ml volume to 5 µg rgD2t in
30 aqueous phase, yielding a 1% oil in water emulsion containing 100 µg 3D-MPL.

Similar adjuvant formulations prepared as above but containing different amounts of rgD2t and/or immunostimulator were used in the
35 second experiment. They were administered in a total volume of 0.5 ml. These formulations are described below.

rgD2t/Alum: Five or 20 µg rgD2t; 0.5 mg equivalents Al^{3+} per 0.5

ml dose.

rgD2t/Alum plus 3D-MPL: Five or 20 µg rgD2t; 0.5 mg equivalents Al³⁺; 50 µg 3D-MPL per 0.5 ml dose.

5

rgD2t/3D-MPL in o/w emulsion (R): Five or 20 µg rgD2t were formulated in an 1% o/w emulsion as described above (2.1.3). A 0.5 ml dose contained 5 µg or 20 µg rgD2t, 50 µg 3D-MPL in a 1% o/w emulsion.

10

rgD2t/3D-MPL in o/w emulsion (S): The vehicle was prepared as follows: To phosphate buffered saline (PBS) containing 0.4% (v/v) Tween 80 are added 5% (v/v) Pluronic L121 and 10% squalane and the resulting mixture microfluidized ten times through a microfluidizer (Model M/110 Microfluidics Corp.,) such that the resulting emulsion comprises only submicron particles. 50µg of 3D-MPL was then added to the emulsion. One volume of this emulsion, containing 3D-MPL was mixed with an equal volume of twice concentrated antigen and vortexed briefly to ensure complete mixing of the components. The final preparation consisted of 0.2% Tween 80, 2.5% Pluronic L121, 5% Squalane, 50µg 3D-Mpl and 5 µg or 20 µg rgD2t in a 0.5 ml dose.

15

20

2.2. Immunization schedule

Groups of female Hartley guinea pigs (200-250 gr) were immunized three times at day 0, 28 and 95 with 5 µg rgD2t formulated in 4 different adjuvant formulations.

25

Immunizations were done subcutaneously with injection volume of 0.25 ml. Control animals were injected according to the same protocol with adjuvant alone or were untreated.

30

The different groups were immunized as follows:

Group 1 (n = 4) : 5 µg rgD2t/3D-MPL (100 µg) in o/w emulsion (R)

Group 2 (n = 4) : 5 µg rgD2t/Alum plus 3D-MPL (100 µg)

Group 3 (n = 4) : 5 µg rgD2t/Alum

35

Group 4 (n = 5) : Alum alone

Group 5 (n = 5) : 3D-MPL (100 µg) alone

Group 6 (n = 8) : untreated

Animals were bled every 2 weeks for antibody determinations by

ELISA and neutralization assays as described below.

The different formulations were also tested for their ability to induce T cell mediated immunity, as measured by the induction of delayed-type hypersensitivity responses. The read-outs applied for evaluation of the humoral and cellular immune responses induced by the different rgD₂t formulations are described below.

In order to compare the protective immunity induced by the rgD₂t formulations, all the guinea pigs were challenged intravaginally with 10⁵ plaque-forming units (pfu) of HSV2, strain MS, 2 weeks after the last immunization. They were monitored daily for clinical signs of acute infection as well as for evidence of recurrent herpetic diseases. Vaginal swab samples were collected on day 5 after viral challenge and titrated for infectious virus.

A detailed description of the guinea pig intravaginal model is given below.

In the second experiment, the immunogenicity of the following rgD₂t formulations was evaluated in larger animal groups. Two antigen doses were compared (5 and 20µg) and different adjuvant composition were tested. A dose of 50µg 3 DMPL was used and its effects compared to the 100µg dose previously used.

25

Groups of female Hartley guinea pigs were immunized three times at days 1, 28 and 84, as follows:

Group I	(n = 8) :	20µg rgD ₂ t/3DMPL (50µg) o/w emulsion (R)
Group II	(n = 8) :	5µg rgD ₂ t/3DMPL (50µg) o/w emulsion (R)
Group III	(n = 10) :	20µg rgD ₂ t/3DMPL (50µg) o/w emulsion (S)
Group IV	(n = 10) :	5µg rgD ₂ t/3DMPL (50µg) o/w emulsion (S)
Group V	(n = 10) :	20µg rgD ₂ t/Alum + 3DMPL (50µg)
Group VI	(n = 10) :	5µg rgD ₂ t/Alum + 3DMPL (50µg)
Group VII	(n = 4) :	Alum + 3DMPL (50µg) alone
Group VIII	(n = 4) :	3DMPL (50µg) o/w emulsion (R) alone
Group IX	(n = 8) :	untreated

Immunizations were given in a 0.5 ml dose. Control groups were immunized according to the same protocol with adjuvant alone (Groups VII and VIII) or were intreated (Group IX).

5

A last group (Group X) was immunized with a gD₂t Alum + 3D-MPL formulation containing 100µg 3D-MPL in a 0.25 ml dose, according to the protocol described in the first prophylactic experiment:

10

Group X (n = 10) : 5µg rgD₂t/Alum plus 3DMPL (100mg).

Animals were bled every two weeks for individual antibody determinations by ELISA and neutralization assays, as described below. Vaginal washings were collected after the second immunization and were
15 assayed for the presence of systemic antibodies specific for gD₂t (anti-gD₂t antibodies of IgG class). Guinea pigs were challenged intravaginally with 105 pfu HSV2 (strain MS) 2 weeks after the last immunization. After challenge, they were monitored daily for clinical signs of acute infection (days 4 to 12 post challenge) as well as for evidence of recurrent
20 herpetic disease (days 13 to 39 post challenge).

3. Read-outs

Several read-outs were set up to evaluate the specific antibody and cell
25 mediated responses induced by vaccination with rgD₂t formulations. The protective value of these formulations was assessed in the guinea pig intravaginal model.

3.1. ELISA

30

An ELISA was designed to detect and quantify gD-specific antibodies in guinea pig sera and vaginal washings, using rgD₂t as the coating antigen.

35 3.1.1. Detection of IgG antibodies specific for rgD₂t in sera

Antigen and antibody solutions were used at 50 µl per well. Antigen was diluted to a final concentration of 1 µg/ml in PBS and was

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adsorbed overnight at 4°C to the wells of 96 wells microtitre plate (Maxisorp Immuno-plate, Nunc, Denmark). The wells were then washed 5 times with PBS Tween 0.1% (wash buffer) and incubated for 1 hour at 37°C with PBS containing 1% bovine serum albumin, 4% newborn calf serum and 0.1% Tween (saturation buffer). Three-fold dilutions of sera (starting at 1/100 dilution) in the saturation buffer were added to the rgD2t-coated wells and incubated for 2 hrs at room temperature. The plates were washed as above and biotin-conjugated sheep anti-guinea pig IgG (IgG1 and IgG2 specific, Serotec, Sopar Biochem., Belgium) diluted 1/3000 in saturation buffer was added to each well and incubated for 1 h.30 min. at 37°C. After a washing step, streptavidin-biotinylated peroxidase complex (Amersham, UK) diluted 1/1000 in saturation buffer was added and incubated for 30 min. at 37°C. Plates were washed as above and incubated with a solution of o-phenylenediamine (Sigma) 0.04% H₂O₂ 0.03% in 0.1 M citrate buffer at pH 4.5. Color reaction was stopped after 15 min by the addition of H₂SO₄ 2 M and the absorbance was readed at 492 nm.

ELISA titer was defined as the reciprocal of serum dilution which produced an absorbance (optical density measured at 492 nm equal to 50% of the maximal absorbance value (midpoint titer).

ELISA titers were calculated by a 4 parameter linear regression analysis using a computer program.

3.1.2. Detection of IgG antibodies specific for rgD2t in vaginal washings

Vaginal washings were first calibrated for their total IgG content by ELISA as follows. Maxisorp Immuno-plates were coated overnight at 4°C with 1 µg/ml (50 µl per well) of purified goat anti-guinea pig IgG (Sigma, Belgium) diluted in PBS. The plates were washed and incubated with saturation buffer as above. Vaginal washings were diluted serially with two-fold dilutions (starting at a 1/100 dilution) in the saturation buffer and added to the plates. A standard curve of purified guinea pig IgG (Sigma, Belgium) was included (two fold dilution starting at a 100 ng/ml concentration) in each plate.

After a 2 hrs incubation at room temperature, the plates were washed as above and biotin-conjugated sheep antibodies specific for guinea pig IgG1 and IgG2 (Serotec, Sopar Biochem, Belgium) diluted

- 10 -

1/1000 in saturation buffer was added to each well and incubated for 1 h 30 min at 37°C. Next steps (addition of streptavidin-biotinylated peroxidase complex and color revelation) were as described above (3.1.1.).

- 5 The concentration of total IgG present in the vaginal washings was determined from the IgG standard curve, by a 4 parameters non-linear regression analysis using a computer program.

- 10 After calibration of their total IgG content, vaginal washings were tested for the presence of IgG antibodies specific for rgD_{2t} using the same ELISA as described for anti-gD antibody sera quantifications. Results were expressed as optical densities measured at 492 nm per 0.5 µg/ml total IgG.

15 3.2. Neutralization assay

A 96 well format neutralization assay was set up as follows:

- 20 Serial two-fold dilutions of the samples to be tested were prepared directly in the 96 W plates (25 µl/well of each serum dilutions, duplicates). Fifty microliters of a mixture containing 4000 pfu of virus HG52 and complement (1/100 final dilution in the well) were added to each well. The plates were incubated for 1 hour at 37°C. One hundred microliters of BHK 21 cell suspension at $4 \cdot 10^5$ cells/ml were then added to
- 25 each well ($4 \cdot 10^4$ cells/well). The plates were centrifuged for 5 minutes at 1000 rpm and incubated for five days at 37°C in the presence of 7% CO₂.

- 30 After this period, the culture medium was gently removed and 100 µl of a solution of cristal violet (10% methanol, 90% H₂O, 0.3% cristal violet) were added to each well and incubated for 20 min. at room temperature. The plates were then abundantly washed with tapwater. The presence of plaques can easily be monitored by microscopic examination.

- 35 The neutralizing titer was defined as the reciprocal of the highest serum dilution at which no viral plaque was observed (100% protection of cytopathogen effect). It is important to note that at this time point, a complete cytopathogen effect (100% lysis of the cell monolayer) was

observed in the control wells.

3.3. Delayed-Type Hypersensitivity (DTH)

5 The different rgD2t formulations were also tested for their ability to induce a T cell specific immune response as measured by the induction of delayed-type hypersensitivity responses.

10 The adjuvant formulations prepared for the first experiment were used in this study. These preparations contained 5 µg of rgD2t per 0.25 ml dose. The immunization schedule was as follows: primary immunization: 0.25 ml of vaccine formulation given intramuscularly; booster immunization: 0.25 ml of vaccine formulation given intramuscularly 21 days later; skin test: 5 µg rgD2t given intradermally
15 (in saline) 8 days later. All guinea pigs were skin tested with saline as control.

 In addition, control guinea pigs (non immunized animals) were skin tested with rgD2t. Erythema and induration at site of intradermal
20 injection were monitored 24 and 48 hrs later.

3.4. Guinea-pig intravaginal model

 The guinea pig model for HSV genital infection has been
25 described by LR Stanberry et al (J. of Infectious Diseases 1982, 146:397-403; Intervirology 1985, 24:226-231).

 Briefly, 2 weeks after the last immunization, the guinea pigs were challenged with 10⁵ pfu of HSV2 strain MS by intravaginal
30 instillation. The clinical course of the primary infection was monitored by daily observation of the incidence and severity of external genital skin lesions during the 12-day post-challenge period.

 Vaginal swabs were collected on day 5 after viral challenge and
35 titered for infectious HSV2 by plaque assay, as described below. Animals were then examined daily for evidence of recurrent herpetic lesions from days 13 to 60. The herpetic lesions on the external genital skin were quantitated by using a lesion score scale ranging from 0 to 4 (0 = no lesion

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or redness; 0.5 = redness; 1 = vesicle; 1.5 = ≥ 4 small vesicles; 2 = larger vesicles; 2.5 = several large vesicles resulting from the fusion of vesicles as in score 2; 3 = size and number of vesicles increase; 3.5 = lesions covering all the surface of the genital skin; 4 = ulcerated lesions with maceration).

5

The degree of protection provided by the different rgD₂t vaccines was evaluated according to the criteria defined below.

Protection against primary disease (days 0 - 12)

10

The animal was considered to be not protected if the following lesions were recorded:

- more than one red area at any time,
- one red area persisting in the same area for at least 3 successive days (0.5 lesion score),
- one or several vesicles (≥ 1 lesion score).

15

Protection against recurrent disease (days 13 - 60)

20

The animal was scored positive for recurrent disease either if a 0.5 lesion score was recorded for 2 successive days at least or if a lesion score ≥ 1 was observed at any day. An episode of recurrent disease was preceded and followed by a day without any lesions or redness.

25

The lesion severity for an animal is calculated as the sum of the scores measured during the primary infection (days 1 - 12). The lesion incidence represents the number of animals showing a lesion of ≥ 1 during the observation period (days 1 - 12 [primary disease] or days 13 - 60 [recurrent diseases]).

30

3.5. Virus titration in vaginal swabs

Vaginal swabs were collected at day 5 after viral challenge. The vaginal vault was swabbed with a calcium alginate tipped swab premoistered in Basal Eagle's medium supplemented with 2% fetal calf serum, 2 mM L glutamine, 100 U/ml penicillin, 100 μ g/ml streptomycin, 100 μ g/ml gentamycin and 1 μ g/ml amphotericin B (swab medium).

35

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Each swab was broken and put into a sterile 12 x 75 mm 5 ml polyallomer tube containing 1 ml of swab medium. The tubes were then vortexed in order to take the virus out and frozen until use. For the titration itself, 6 wells culture plates containing $5 \cdot 10^5$ cells /well were incubated overnight at 37°C. The tubes were thawed and serial dilutions of the samples in swab medium were prepared. After removal of the culture medium in the 6 wells, 200 µl of each samples dilution were transferred in duplicate on the cell monolayers and kept for one hour at 37°C. Four ml of a culture medium containing 1.5% carboxymethylcellulose were added to each well. The plates were then incubated for 2 days at 37°C. After this incubation period, the medium was gently removed and 1 ml of a solution of cristal violet (10% methanol, 90% H₂O, 0.3% cristal violet) was added to each well for 15 min. The plates were then thoroughly rinsed and the plaques were counted. HSV2 titer was expressed in pfu/ml.

4. Results

In a first set of experiments, groups of guinea pigs were immunized with a low antigen dose (5 µg rgD₂t) formulated in 4 different formulations. This suboptimal antigen dose was chosen in order to select the more potent rgD₂t adjuvant combination that could provide protection against primary and recurrent HSV disease when administered to guinea pigs prior to intravaginal HSV2 inoculation (prophylactic trials).

4.1. Induction of humoral immunity

As shown in Table 1, groups vaccinated with rgD₂t formulations containing 3D-MPL as immunostimulant showed higher ELISA and neutralizing titers in their sera than the group immunized with the rgD₂t/Alum vaccine. Good mean neutralizing titers were induced after 3 immunizations with rgD₂t 3D-MPL o/w (R) or rgD₂t Alum 3D-MPL.

4.2. Induction of effector T cell response (DTH)

Skin test results (Table 2) showed that rgD₂t formulated in 3D-MPL o/w emulsion induced the strongest DTH response. A specific DTH response was also induced by rgD₂t Alum 3D-MPL. Similar experiments

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conducted in mice also revealed that rgD₂t combined with Alum plus 3D-MPL was very potent in inducing an in vivo effector T cell response, in contrast to rgD₂t Alum formulation.

5 4.3. Effect of vaccination on HSV primary disease

Two weeks after the third immunization, guinea pigs were challenged intravaginally with HSV2. The effect of vaccination on the clinical and virological course of primary HSV2 infection is illustrated in
10 Figure 1 and summarized in Table 3. As compared to the control groups (Groups 4 to 6) that became infected and experienced acute primary disease, 100% of the animals vaccinated with the rgD₂t 3D-MPL o/w formulation showed no evidence of herpetic disease, as monitored by skin lesion incidence and severity. Moreover, these animals did not show any
15 viral replication in the vaginal tract as determined by vaginal virus titration at day 5 post challenge. Very similar results were obtained in the group vaccinated with rgD₂t/Alum 3D-MPL. This group never developed herpetic vesicles during the observation period (lesion score < 1). Moreover, very low viral replication could be detected in the vaginal
20 swabs collected. In contrast animals rgD₂t adsorbed on alum were poorly protected (75% skin lesion incident).

4.4. Effect of vaccination on HSV recurrent disease

25 Results are illustrated in Figure 1 and summarized in Table 4.

Vaccination with rgD₂t formulations containing 3D-MPL (Groups 1 and 2) significantly altered the development of recurrent herpetic diseases. Two groups had significantly fewer recurrent episodes and
30 recurrent day numbers than control or rgD₂t Alum treated groups.

In order to further evaluate the factors influencing the efficacy of prophylactic rgD₂t vaccines containing 3DMPL, a second set of experiments was initiated on larger guinea pig numbers.

35

Two antigen doses were compared (5 and 20µg) and different adjuvant compositions were tested. Three immunizations were administered at days 0, 28 and 84. Animals were bled every two weeks for individual

antibody determination by ELISA and neutralization assays. Vaginal washings were collected after the second immunization and were tested for the presence of systemic antibodies specific for rgD2t.

5 Induction of humoral immunity

Results (Table 5) indicated that all the rgD2t formulations containing 3D-MPL were able to stimulate high ELISA and neutralizing titers in the guinea pig sera.

10

The mean ELISA and neutralizing titers induced after three immunizations were very similar in the sera of groups vaccinated with a rgD2t formulation containing either 5µg or 20µg gD2t. There was no significant difference in the humoral response measured in the groups immunized with a rgD2t Alum vaccine containing either 50µg 3D-MPL (Group VI) or 100mg 3D-MPL (Group X).

15

It is interesting to note that systemic anti-rgD2t antibodies (IgG class) could be detected in the vaginal washings of all vaccinated groups. This mucosally located anti-rgD2t antibody response may play an important protective role by decreasing the load of infectious virus in the genital tract during primary infection.

20

Effect of vaccination on HSV primary disease

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Two weeks after the third immunization, guinea pigs were challenged intravaginally with HSV2. The effect of vaccination on the clinical and virological course of primary HSV2 infection is summarized in Table 6. As compared to the controls, animals vaccinated with a 5µg rgD2t Alum 3D-MPL formulation containing either 50µg or 100µg 3D-MPL (Groups VI and X) showed significantly ($p < 0.05$) reduced skin lesion severity as well as reduction of skin lesions incidence.

30

Very similar results were observed in the group vaccinated with 5µg rgD2t in a 3D-MPL o/w emulsion (Group III). In the three vaccinated groups, very low viral replication could be detected in the vaginal swabs collected 5 days after the challenge.

35

Effect of vaccination on HSV recurrent disease

Results are given in Table 6. As compared to the control groups, the incidence of skin lesions and the recurrence day number were significantly ($p > 0.05$) reduced in the three vaccinated groups. These groups had also fewer recurrent episodes than control groups.

5. Conclusions

Results obtained in guinea pigs clearly show that vaccination with a rgD₂t formulation containing 3D-MPL delivered in an oil in water emulsion or combined with aluminium hydroxyde is very effective in providing protection against primary and recurrent HSV2 disease when administered to guinea pigs prior to HSV2 inoculation. Such rgD₂t 3D-MPL formulations are able to improve specific humoral (neutralizing antibodies) and effector cell mediated (DTH) immune responses. These results are obtained using a low dose of rgD₂t (5µg).

6. Immunogenicity of gD₂t formulations in primates

6.1 Comparative immunogenicity of rgD₂t/Alum and rgD₂t/Alum 3D-MPL form

The immunogenicity of rgD₂t/Alum and rgD₂t/Alum 3D-MPL vaccines were evaluated in cercopithecus aethiops (African Green Monkeys, AGM). Three immunizations were given at 0, 1 and 3 months. Specific humoral (ELISA and neutralizing titers) and effector cell mediated (DTH) immune responses were measured.

6.1.1. Experimental procedure

Each formulation contained 20mg rgD₂t and 0.5mg equivalents AL₃+/dose. A dose of 50µg 3D-MPL was used. Groups of cercopithecus aethiops (AGM) were immunized 3 times at days 0, 28 and 84. Immunizations were given intramuscularly in a 0.5ml dose (20 rgD₂t). Animals were bled every \pm 2 weeks for antibody determination by ELISA and neutralization assays. The two formulations were also tested for their ability to induce T cell mediated immunity, as measured by the induction of delayed-type hypersensitivity (DTH) responses. Monkeys were given

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intradermally on the belly different rgD₂t doses (20, 5 and 1µg) in saline 13 days after the second immunization. They were also skin tested with saline alone as control. Erythema and induration at site of intradermal injection were monitored 24 hrs and 48 hrs later.

5

6.1.2. Results

a) Induction of humoral immunity

Before vaccination, none of the monkey sera showed any
10 anti-HSV2 antibody activity (data not shown). As shown in table 7, both vaccines induced good ELISA and neutralizing titers after the second immunization. This antibody response was not boosted with a third immunization in the rgD₂t/Alum vaccinated monkeys. In contrast, monkeys receiving a third immunization with rgD₂t/Alum 3D-MPL
15 produced increased ELISA and neutralizing antibody responses (mean ELISA titer: 10056; mean neutralizing titer: 950).

b) Induction of effector T cell response (DTH)

Skin test results (table 8) showed that rgD₂t combined with Alum plus 3D-MPL was very potent in inducing an *in vivo* effector T cell
20 response, in contrast to the rgD₂t Alum formulation. A strong DTH response was observed in all rgD₂t Alum 3D-MPL vaccinated animals skin tested with 20mg rgD₂t. Specific DTH responses were also measured with the lower gD₂t concentrations (5 and 1µg) in the majority of the monkeys (3/4 for the 5µg dose and 2/4 for the 1µg dose). These rgD₂t
25 doses induced weaker skin test responses than the 20mg rgD₂t concentration.

6.2. Immunogenicity of rgD₂t/Alum 3D-MPL formulations in rhesus monkeys

30

The immunogenicity of rgD₂t/Alum 3D-MPL vaccines containing different rgD₂t doses (100µg, 10µg, or 5µg) was compared in rhesus monkeys.

6.2.1. Experimental procedure

35

Each formulation contained 0.5µg equivalents Al³⁺ and 50µg 3D-MPL per dose. Three groups of rhesus monkeys (4 monkeys/group) were immunized three times at days 0, 28 and 77, as follows:

- Group 1 : 100µg rgD₂t Alum plus 3D-MPL (50µg)
Group 2 : 20µg rgD₂t Alum plus 3D-MPL (50µg)
Group 3 : 5µg rgD₂t Alum plus 3D-MPL (50µg)

5

Immunizations were given intramuscularly in a 1 ml dose. Animals were bled every \pm 2 weeks for antibody determination by ELISA and neutralization assays.

10 6.2.2. Induction of humoral immunity

Before vaccination, none of the monkey sera showed any anti-HSV2 antibody activity. Good ELISA and neutralizing titers were observed in the three vaccinated groups receiving either 100, 20 and 5mg
15 gD₂t in Alum + 3D-MPL. (Data not shown).

6.3. Conclusions

Results obtained in cercopithecus aethiops clearly indicate that a rgD₂t
20 vaccine containing a combination of Alum with 3D-MPL significantly improve humoral (neutralizing antibodies) and effector cell mediated (DTH) specific immune responses. As compared to this vaccine, a rgD₂t Alum formulation is less potent in inducing neutralizing antibodies and is unable to induce an *in vivo* DTH response.

25

Results obtained in rhesus monkeys also show that a rgD₂t Alum + 3D-MPL formulation is very effective in inducing a specific humoral response, even with low doses of antigen (5µg or 20µg rgD₂t).

30 7. General Conclusions

Results obtained in guinea pigs clearly indicate that adjuvant formulations containing either 3D-MPL delivered in an oil in water emulsion or combined with aluminium hydroxide are very effective in
35 inducing a protective immune response with a recombinant HSV glycoprotein vaccine in the intravaginal guinea pig challenge animal model, even with very low doses of antigen (5 µg rgD₂t). Protection data also show that these rgD₂t 3D-MPL formulations are more potent in

providing protection. Such 3D-MPL formulations are able to improve specific humoral (neutralizing antibodies) and effector cell mediated (DTH) immune responses.

- 5 Furthermore, the rgD₂t Alum 3D-MPL formulation was shown to also improve immunogenicity at the antibody level and to induce an effector T cell response in primates, suggesting that this adjuvant effect is not restricted to small animal species.

TABLE 1 : Anti-HSV antibody response in sera of guinea pigs immunized with rgD₂t formulations before and after viral challenge.

Group	Vaccine (1)		Pre-challenge (2)		Post-challenge (3)	
	Antigen	Adjuvant	ELISA titer	Neutralizing titer	ELISA titer	Neutralizing titer
1	rgD ₂ t	3D-MPL o/w (R)	81291 ± 20822	1600	68720 ± 24648	2200 ± 765
2	rgD ₂ t	Alum 3D-MPL	39897 ± 30165	2000 ± 800	27224 ± 13093	1800 ± 765
3	rgD ₂ t	Alum	20346 ± 23704	600 ± 400	28622 ± 24024	1333 ± 461
4	-	Alum	< 100	< 50	737 ± 878	142 ± 85
5	-	3D-MPL	< 100	< 50	259 ± 244	1275 ± 1304
6	untreated	-	< 100	< 50	225 ± 194	119 ± 141

- (1) rgD₂t dose = 5 µg. Animals were immunized three times at days 0, 28 and 95. They were challenged 2 weeks later with 10⁵ pfu HSV2.
- (2) Sera collected the day before challenge (= 14 days after the third immunization)
- (3) Sera collected 2 weeks after challenge.

Values are given as arithmetic mean titers ± SD.

TABLE 2 : Skin Test Results (DTH) in guinea pigs vaccinated with rgD_{2t} formulations.

Formulation	Guinea Pig #	24 hr reading		48 hr reading	
		E (mm)	E (mm)	E (mm)	I (mm)
rgD _{2t} 3D-MPL α/w (R)	1	20	15	14	10 (N)
	2	15	10	10	3
	3	20	17 (N)	15	12 (N)
rgD _{2t} Alum 3D-MPL	1	10	8	10	4
	2	15	12	12	3
	3	11	9	12	0
- Alum 3D-MPL	1	0	0	0	0
	2	0	0	0	0
	3	0	0	0	0
untreated	1	0	0	0	0
	2	0	0	0	0

Guinea pigs were immunized at days 0 and 21 with 5 µg rgD_{2t} formulation (given intramuscularly). They were given intradermally 5 µg rgD_{2t} in saline at day 29. Skin test was read at 24 h and 48 h.
 E = erythema at site of ID injection in millimeters.
 I = induration at site of ID injection in millimeters.
 N = necrosis at skin test site.

TABLE 3 : Effect of immunization with rgD₂t formulations on the clinical and virological course of primary HSV2 infection in guinea pigs.

Group	Vaccine (1)		Incidence of Skin Lesions (2)	Skin Lesion Severity (3)	Vaginal Virus Titers (4)
	Antigen	Adjuvant			
1	rgD ₂ t	3D-MPL o/w (R)	0/4	0.1 ± 0.3	0
2	rgD ₂ t	Alum 3D-MPL	0/4	1 ± 0.4	6.25 ± 12.4
3	rgD ₂ t	Alum	3/4	4.4 ± 2.7	3575 ± 6010
4	-	Alum	5/5	6.2 ± 2.6	5216 ± 6295
5	-	3D-MPL	4/5	5.1 ± 3.6	3298 ± 4475
6	untreated	-	7/8	7.3 ± 4.7	2214 ± 4519

- (1) rgD₂t dose = 5 µg. Animals were immunized three times at days 0, 28 and 95. They were challenged 2 weeks later with 10⁵ pfu HSV2.
- (2) Number animals showing a lesion score ≥ 1 during the 12 days observation period
- (3) Sum of the lesion scores (days 1 - 12), arithmetic mean ± SD
- (4) Peak HSV titer (pfu/ml) in vaginal swabs collected 5 days post challenge.

TABLE 4 : Effect of immunization with rgD₂t formulations on the recurrent genital HSV2 disease in guinea pigs.

Group	Vaccine (1)		Incidence of Skin Lesions (2)	Episodes of recurrent disease (3)	Recurrence (4) days Numbers
	Antigen	Adjuvant			
1	rgD ₂ t	3D-MPL o/w (R)	1/4	1 ± 2	0.7 ± 1.5
2	rgD ₂ t	Alum 3D-MPL	2/4	1 ± 0.8	1.7 ± 3.5
3	rgD ₂ t	Alum	3/3	4.3 ± 1.5	8.3 ± 5
4	-	Alum	4/5	3.8 ± 3.3	7.6 ± 6.5
5	-	3D-MPL	5/5	2.6 ± 1.1	6 ± 4.4
6	untreated		6/8	3.5 ± 2.2	9.9 ± 6

- (1) rgD₂t dose = 5 µg. Animals were immunized three times at days 0, 28 and 95. They were challenged 2 weeks later with 10⁵ pfu HSV2.
- (2) Number animals showing a lesion score ≥ 1 during the observation period (days 13 - 60)
- (3) One recurrent episode is preceded and followed by a day without lesion and characterised by at least two days with erythema (score = 0.5) or one day with vesicle(s) (lesion score ≥ 1). Results expressed as arithmetic mean ± SD (observation period: days 13-60).
- (4) Total days animals experienced a recurrent/herpetic episode, arithmetic mean ± SD (observation period: days 13-39).

TABLE 5 : COMPARISON OF THE EFFECT OF DIFFERENT ADJUVANT FORMULATIONS ON THE IMMUNOGENICITY OF γ D2t IN GUINEA PIGS

GROUP	rgD2t dose	VACCINE (1)		Anti-HSV antibody response after two immunisations		Anti HSV antibody response after three immunisations - prechallenge titer (3)	
		Adjuvant		IN SERA	IN VAGINAL (5)	IN SERA (4)	
				Elisa titer	Neutral titer	Elisa titer	Neutral titer
I	20 μ g	3DMPL (50 μ g) o/w (R)		31462 \pm 9087	850 \pm 396	19958 \pm 10171	3400 \pm 1994
II	5 μ g	3DMPL (50 μ g) o/w (R)		35015 \pm 14395	412 \pm 264	51688 \pm 40120	4342 \pm 2879
III	20 μ g	3DMPL (50 μ g) o/w (S)		16720 \pm 12641	1380 \pm 756	36647 \pm 24126	4080 \pm 2883
IV	5 μ g	3DMPL (50 μ g) o/w (S)		14992 \pm 9885	840 \pm 571	45082 \pm 24221	4560 \pm 2502
V	20 μ g	Alum 3DMPL (50 μ g)		14452 \pm 7476	740 \pm 499	16015 \pm 7846	3280 \pm 2276
VI	5 μ g	Alum 3DMPL (50 μ g)		10174 \pm 4219	420 \pm 301	20488 \pm 9562	2840 \pm 1510
VII	-	Alum 3DMPL (50 μ g)		<100	<50	<100	<50
VIII	-	3DMPL (50 μ g) o/w (R)		<100	<50	<100	<50
IX	-	untreated		<100	<50	<100	<50
X	5 μ g	Alum 3DMPL (100 μ g)		4602 \pm 3953	163 \pm 151	16588 \pm 6945	2560 \pm 1678

(1) Animals were immunized three times at days 0, 28 and 84. They were challenged 2 weeks later with 10^5 pfu HSV2.

(2) Sera and vaginal washings collected 14 days after the second immunization.

(3) Sera collected the day before challenge (= 14 days after the third immunization).

(4) Values are given as arithmetic mean titers \pm SD

(5) Corresponds to the optical density (at 492 nm) per 0.5 μ g/ml total IgG measured in the standard anti-gD2t ELISA assay, arithmetic mean \pm SD.

TABLE 6 : EFFECT OF IMMUNIZATION WITH rgD2t FORMULATIONS ON THE CLINICAL AND VIROLOGICAL COURSE OF HSV2 INFECTION IN GUINEA PIGS

	VACCINE				CONTROLS
	5µg rgD2t 3DMPL o/w (s) Group III	5µg rgD2t Alum 3DMPL(50µg) Group VI	5µg rgD2t Alum 3DMPL(100µg) Group X	Groups VII - VIII - IX	
PRIMARY HSV2 INFECTION					
Incidence of skin lesions (%)	1/9 11%	1/10 10%	0/10 0%	12/14 86%	
Skin lesion severity	1.2 ± 1	0.7 ± 0.7	0.9 ± 1	8.6 ± 5.1	
Vaginal virus titers (pfu/ml)	0	0	1.5 ± 4.7	1077 ± 1682	
RECURRENT HSV2 INFECTION					
Incidence of skin lesions (%)	2/9 22%	2/10 20%	3/10 30%	11/14 79%	
Recurrence day number	1 ± 2.3	1.6 ± 2.1	1.6 ± 2.7	7.3 ± 6	
Recurrence episode number	0.2 ± 0.4	0.6 ± 0.7	0.5 ± 0.8	1.9 ± 1.2	

Experimental schedule : 3 immunizations at days 0, 28 and 84.

Challenge 2 weeks after the last immunization with 10⁵ pfu HSV2.

Primary HSV2 infection :

Incidence of skin lesions (%) :

Skin lesion severity :

Vaginal virus titers :

(observation period days 4 to 12 post challenge)

number of animals with vesicle(s) (lesion score ≥ 1)

sum of the lesion scores (for the days 4 to 12), arithmetic mean ± SD

virus titers (pfu/ml) in vaginal swabs collected 5 days after the challenge

Recurrent HSV2 infection :

Incidence of skin lesions (%) :

Recurrence day number :

(observation period days 13 to 39 post challenge)

number (%) of animals with vesicle(s) (lesion score ≥ 1)

total days animals experienced a recurrent herpetic disease, arithmetic mean ± SD. Animals were scored positive for recurrent disease either if a 0.5 lesion score (erythema) was recorded for 2 successive days at least or if a lesion score ≥ 1 (vesicle(s)) was observed at any day.

Recurrence episode number : arithmetic mean, ± SD

TABLE 7

DTH RESULTS IN AFRICAN GREEN MONKEYS VACCINATED WITH GD2t ALUM OR GD2t ALUM 3D MPL

VACCINE	MONKEY	24 h reading				48 h reading			
		PBS	GD2t	GD2t	GD2t2	PBS	GD2t	GD2t	GD2t
	NB	1 µg	5 µg	0 µg	0 µg	1 µg	5 µg	20 µg	20 µg
GD2t	JO358	-	ND	-	-	-	ND	-	-
ALUM	JO359	-	ND	-	-	-	ND	-	-
	JO363	-	ND	-	-	-	ND	-	-
	JO364	-	ND	-	-	-	ND	-	-
	JO366	-	ND	-	-	-	ND	-	-
GD2t	JO348	-	-	E	I 2-4	-	-	I	I
ALUM	JO349	-	E 1-2	I 5-8	cm	-	E	I	I
3D MPL	JO375	-	mm	mm	E 7-9	-	E	I	I
	JO515	-	E 1-2	I 3-4	mm	-	-	-	Eweak
			mm	mm	I 4-6				
			-	-	mm				
					E				
CONTROLS	JO320	-	-	-	-	-	-	-	-
	JS110	-	-	-	-	-	-	-	-

Monkeys were immunized at days 0 and 28 with 20 µg GD2t formulation (given intramuscularly). They were given intradermally in the belly different GD2t doses in saline 13 days later. Skin test was read at 24 h and 48 h.

E : erythema at site of ID injection

I : induration at site of ID injection

ND = not done

TABLE 8

COMPARATIVE IMMUNOGENICITY OF GD2T ALUM AND GD2T ALUM 3D MPL FORMULATIONS IN AFRICAN GREEN MONKEYS:
SEROLOGICAL RESPONSES

VACCINE*	MONKEY NB	Post II				Post III	
		14 days		28 days		56 days	
		ELISA TITER	NEUT TITER	ELISA TITER	NEUT TITER	ELISA TITER	NEUT TITER
GD2t ALUM	JO 358	1388	400	6572	200	2050	400
	JO 359	4731	400	3232	100	2110	200
	JO 363	1376	200	2316	50	1205	50
	JO 364	5914	1600	5275	400	6323	800
	JO 366	21104	400	3696	200	2302	200
	<i>Arit mean ± SD</i>	<i>6902 ± 8190</i>	<i>600 ± 565</i>	<i>4218 ± 1697</i>	<i>190 ± 134</i>	<i>2798 ± 2015</i>	<i>330 ± 290</i>
GD2t ALUM/ 3D MPL	JO 348	7120	200	10175	200	11082	400
	JO 349	14437	1600	15409	800	15848	1600
	JO 375	7990	800	5170	800	6797	1600
	JO 515	6515	200	7246	100	6497	200
	<i>Arit mean ± SD</i>	<i>9015 ± 3664</i>	<i>700 ± 663</i>	<i>9500 ± 4442</i>	<i>475 ± 377</i>	<i>10056 ± 4392</i>	<i>950 ± 754</i>

* Each vaccine dose contains 20 µg GD2t

ELISA titer = midpoint titer

NEUT titer = reciprocal of the highest serum dilution giving 100% protection against the cytopathogen effect

Claim

1. A vaccine formulation comprising an HSV glycoprotein D or an immunological fragment thereof in conjunction with 3Deacylated monophosphoryl lipid A and a suitable carrier.
2. A vaccine formulation as claimed in claim 1 wherein the carrier is alum.
3. A vaccine formulation as claimed in claim 2 wherein the carrier is an oil in water emulsion.
4. A vaccine formulation as claimed in any of claims 1 to 3 wherein the glycoprotein D is an HSV-2 glycoprotein D or immunological fragment thereof.
5. A vaccine formulation as claimed in claim 1 to 4 wherein the glycoprotein D is a truncated protein.
6. A vaccine formulation as claimed in claim 5 wherein the truncated protein is HSVgD₂ and is devoid of the C terminal anchor region.
7. A vaccine formulation as claimed herein wherein the glycoprotein D is conjuncted to a particulate carrier.
8. A vaccine formulation as claimed herein wherein 3Deacylated monophosphoryl lipid A is present in the range of 10µg - 100µg per dose.
9. A vaccine formulation as claimed herein for use in medicine.
10. Use of HSV glycoprotein gD or an immunological fragment thereof in conjunction with 3Deacylated monophosphoryl lipid A in the manufacture of a medicament for the prophylaxis or treatment of HSV infections.
11. A method of treating a human subject suffering from or susceptible to Herpes Simplex infections comprising administering an effective amount of a vaccine according to any one of claims 1 to 8.

12. A method of producing a vaccine according to any of claims 1 to 8 wherein the method comprises mixing HSV glycoprotein D or immunological fragment with a carrier and 3Deacylated monophosphoryl lipid A.

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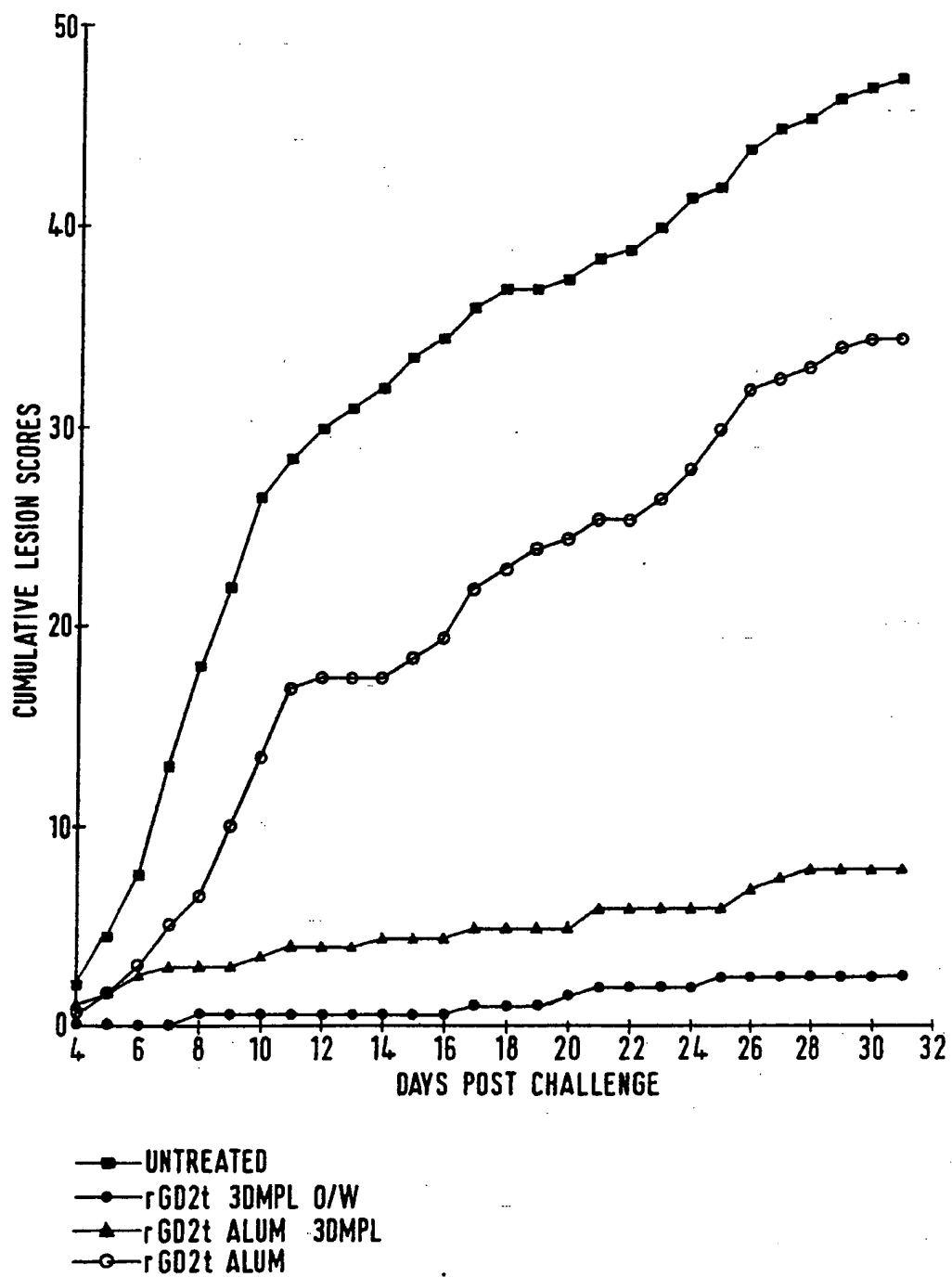


Fig.1

INTERNATIONAL SEARCH REPORT

International App

No

PCT/EP 92/00592

I. CLASSIFICATION OF SUBJECT MATTER (If several classification symbols apply, indicate all)

According to International Patent Classification (IPC) or to both National Classification and IPC
 Int.C1.5 A 61 K 39/245

II. FIELDS SEARCHED

Minimum Documentation Searched?

Classification System

Classification Symbols

Int.C1.5

A 61 K

C 07 K

Documentation Searched other than Minimum Documentation
 to the Extent that such Documents are Included in the Fields Searched⁸

III. DOCUMENTS CONSIDERED TO BE RELEVANT⁹

Category ¹⁰	Citation of Document, ¹¹ with indication, where appropriate, of the relevant passages ¹²	Relevant to Claim No. ¹³
Y	WO,A,8802634 (CHIRON CORP.) 21 April 1988, see the whole document, especially pages 42-43 ---	1-12
Y	GB,A,2220211 (RIBI IMMUNOCHEM. RESEARCH INC.) 4 January 1990, see the whole document (cited in the application) ---	1-12
A	EP,A,0356340 (THE LIPSOME CO., INC.) 28 February 1990, see the whole document ---	1-12
A	EP,A,0139417 (GENENTECH, INC.) 2 May 1985, see pages 26-31d (cited in the application) -----	1-12

¹⁰ Special categories of cited documents:

- "A" document defining the general state of the art which is not considered to be of particular relevance
- "E" earlier document but published on or after the international filing date
- "L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
- "O" document referring to an oral disclosure, use, exhibition or other means
- "P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"A" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search

21-05-1992

Date of Mailing of this International Search Report

3 0. 06. 92

International Searching Authority

EUROPEAN PATENT OFFICE

Signature of Authorized Officer

Miss MORTENSEN

FURTHER INFORMATION CONTINUED FROM THE SECOND SHEET

V. ☒ OBSERVATION WHERE CERTAIN CLAIMS WERE FOUND UNSEARCHABLE ¹

This International search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☒ Claim numbers because they relate to subject matter not required to be searched by this Authority, namely:
Remark: Although claim 11 is directed to a method of treatment of the human body the search has been carried out and based on the alleged effects of the composition.
2. ☐ Claim numbers because they relate to parts of the International application that do not comply with the prescribed requirements to such an extent that no meaningful International search can be carried out, specifically:
3. ☐ Claim numbers because they are dependant claims and are not drafted in accordance with the second and third sentences of PCT Rule 6.4(a).

VI. ☐ OBSERVATIONS WHERE UNITY OF INVENTION IS LACKING ²

This International Searching Authority found multiple inventions in this International application as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this International search report covers all searchable claims of the International application
2. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims of the International application for which fees were paid, specifically claims:
3. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claim numbers:
4. ☐ As all searchable claims could be searched without effort justifying an additional fee, the International Searching Authority did not invite payment of any additional fee.

Remark on Protest

- ☐ The additional search fees were accompanied by applicant's protest.
☐ No protest accompanied the payment of additional search fees.

**ANNEX TO THE INTERNATIONAL SEARCH REPORT
ON INTERNATIONAL PATENT APPLICATION NO.**

EP 9200592
SA 57226

This annex lists the patent family members relating to the patent documents cited in the above-mentioned international search report. The members are as contained in the European Patent Office EDP file on 12/06/92. The European Patent Office is in no way liable for these particulars which are merely given for the purpose of information.

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO-A- 8802634	21-04-88	EP-A- 0289550	09-11-88
		JP-T- 1500999	06-04-89
GB-A- 2220211	04-01-90	US-A- 4912094	27-03-90
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		JP-A- 60155128	15-08-85